RESEARCH ARTICLE

Factors affecting adventitious regeneration from *in vitro* leaf explants of 'Improved French' plum, the most important dried plum cultivar in the USA

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Abstract

An adventitious shoot regeneration protocol from *in vitro* leaves of the most important dried plum cultivar in the USA, 'Improved French', has been established. Factors affecting regeneration were studied in order to optimise regeneration. The proliferation medium in which the shoots, used as the source of leaf explants, were cultured had a strong influence on subsequent regeneration. Shoot regeneration was observed at a mean frequency of 52% when a Murashige-based and Skoog-based shoot culture medium with 3 µM N^6-benzylaminopurine and 0.25 µM indole-3-butyric acid (IBA) was employed compared with shoot regeneration frequencies of less than 5% for a Quoirin-based and Lepoivre-based shoot culture medium, with 8.9 µM N^6-benzylaminopurine and 0.49 µM IBA. The shoot regeneration medium contained α-naphthaleneacetic acid at 2.0–6.0 µM and thidiazuron at 4.5–15.0 µM. 2,4 Dichlorophenoxy-acetic acid at 9.0 µM was included in the medium but only for the first 4 days of culture. Shoot regeneration frequencies were positively related to thidiazuron concentration and significantly greater (P < 0.05) for 9–15 µM thidiazuron than for the media with 4.5 µM thidiazuron. Leaf explants, incubated in a 16-h-light/8-h-dark photoperiod or in the dark for 1 week followed by exposure to light, showed significantly more organogenic activity (P < 0.01) than was observed for leaves cultured in the dark for 2 or 3 weeks before they were transferred to the light. The utilisation of Bacto agar (0.7%) as the gelling agent increased organogenesis compared with media gelled with TC Agar (0.7%), or an agar–gellan gum blend (Agargel^{TM}) (0.45%). The addition of the ethylene inhibitor silver thiosulphate at 60–120 µM also improved organogenesis. When all the studied factors were optimised, a regeneration rate of 65% was achieved. Rooting frequency of regenerated shoots was significantly increased (P < 0.05) by the use of full-strength Murashige and Skoog salts (40%) or 100 mg L^{-1} phloroglucinol (53%) to the rooting medium.

Introduction

'Improved French', also known as 'French' or 'French Prune', is perhaps the most economically important European plum (*Prunus domestica* L.) in the world. It is particularly suited to drying and accounts for most of the world's trade in dried plums (prunes). 'Improved French' is particularly important in California where it accounts for 99% of the dried plum production, which reached 180 000 tons in 2006 with a production value of US $255 684 000 (USDA, 2006). California supplies 100% of

the USA and 60% of the world's dried plums (Boriss & Brunke, 2005). The origin of 'Improved French' is 'Prune d'Agen' imported from France in the mid-1800s (Hedrick, 1911). 'Improved French' is probably a seedling of 'Prune d'Agen' that was selected by Luther Burbank (J. Doyle, personal communication). 'Improved French' is similar to 'Prune d'Agen' in appearance and quality. 'Prune d'Agen' and the many budsports of 'Prune d'Agen' form the basis of the dried plum industry in France, which is the second largest producer after California (FAS, 2005). Virtually, all dried plums in world trade are 'Improved French' or budsports of 'Prune d'Agen', and new varieties must meet or exceed the quality standards of this closely related germplasm.

Conventional plum breeding, similar to breeding temperate fruit trees in general, is constrained by a long reproductive cycle, complex reproductive biology (such as self-sterility), polyploidy and a high degree of heterozygosity (Okie & Weinberger, 1996; Petri & Burgos, 2005). Trait improvements in plum such as delayed bloom, changes in ripening date and disease resistance must be developed within a defined set of quality parameters that include specific industrial and consumer-oriented traits such as sugar level, skin and flesh colour, pit size, flesh adhesion to the stone and moisture content for drying. As opposed to conventional breeding, a potential advantage of genetic engineering (GE) lies in the ability to target specific improvements in established cultivars. Genetic improvement through GE will likely require less time, labour and field space (Petri & Scorza, 2008). Fixation through the sexual cycle is unnecessary because cultivars are clonally propagated. In addition, the targeted improvement of established cultivars avoids the time and effort necessary for the establishment of a new cultivar in the market. Because most of the production of dried plums in the USA is based on 'Improved French' (USDA, 2006), the potential impact of genetic engineering this cultivar is significant.

For most plant GE protocols, adventitious shoot regeneration is one of the most important rate limiting steps. Fruit trees are especially recalcitrant for shoot regeneration (Petri & Burgos, 2005). Moreover, regeneration efficiency is highly genotype dependent and in some cases, regeneration protocols cannot be transferred between cultivars. There are few reports on *in vitro* adventitious shoot regeneration from clonal explants of European plum. In these reports, regeneration rates are low, 12–32% depending on the genotype (Bassi & Cossio, 1991; Escalettes & Dosba, 1993; Csányi *et al.*, 1999), or the varieties used as explant sources are generally of limited or of local importance (Nowak *et al.*, 2004; Mikhailov & Dolgov, 2007). In addition, none of these prior research groups worked with the 'Improved French' cultivar.

The present study was designed to explore the conditions required for the regeneration of shoots from leaf explants of 'Improved French' plum, and to maximise regeneration. Regeneration was investigated using a strategy that was successfully applied to regenerate apricot (*Prunus armeniaca* L.) from leaf explants (Petri *et al.*, 2008a). Parameters shown to generally affect shoot regeneration in fruit tree species were evaluated, including shoot proliferation medium (Burgos & Alburquerque, 2003), light (Leblay *et al.*, 1990; Korban *et al.*, 1992; Miguel *et al.*, 1996; Pérez-Tornero *et al.*, 2000; Gentile *et al.*, 2002; Espinosa *et al.*, 2006), gelling agent (Welander & Maheswaran, 1992; Chevreau *et al.*, 1997; Pérez-Tornero *et al.*, 2000; Burgos & Alburquerque, 2003) and growth regulators (Escalettes & Dosba, 1993; Declerck & Korban, 1996), including ethylene inhibitors (Escalettes & Dosba, 1993; Burgos & Alburquerque, 2003; Petri *et al.*, 2005).

Materials and methods

Establishment of shoots *in vitro* and maintenance of cultures

Young 10–15 cm long shoots from current season growth of 1 to 2-year-old greenhouse-grown 'Improved French' plum were the source of explants for establishing the *in vitro* cultures. Leaves were removed and shoots were cut into 1.5–2.0 cm long sections, each with one or two nodes. Sections were washed in deionised water with 0.025% LiquiNox® (Alconox, White Plains, NY, USA) for 5 min, and then rinsed three times with sterile deionised water. For surface disinfestation, the explants were immersed for 2 h in an aqueous solution of 0.01 M sodium dichloroisocyanurate (Sigma, St Louis, MO, USA) (Parkinson *et al.*, 1996). Explants were then rinsed three times with sterile deionised water and placed in shoot multiplication media. As shoots developed from the nodes of the stem explants, they were removed and subcultured to fresh medium. Every 3 weeks, new axillary shoots (>1 cm tall) were harvested and individually subcultured in fresh medium.

Two media, used previously with *P. domestica*, were used for shoot multiplication: JS, as described by Mikhailov & Dolgov (2007) or shoot growth medium (SGM), as described by Gonzalez-Padilla *et al.* (2003). JS medium consisted of Quoirin and Lepoivre (QL) salts (Quoirin & Lepoivre, 1977) supplemented with 100 mg L^{-1} myoinositol, 0.1 mg L^{-1} thiamine HCl, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl, 2.0 mg L^{-1} glycine, 3% (w/v) sucrose, 8.89 μM N^6-benzylaminopurine (BA) and 0.49 μM indole-3-butyric acid (IBA). SGM consisted of three fourth strength of Murashige and Skoog (MS) salts (Murashige &

Skoog, 1962) supplemented with 100 mg L^{-1} myoinositol, 0.1 mg L^{-1} thiamine HCl, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine HCl, 2.0 mg L^{-1} glycine, 2% sucrose (w/v), 3.0 µM BA and 0.25 µM IBA.

Bacto agar (Becton, Dickinson and Company, Sparks, MD, USA; Cat. No. 214010) at 0.7% (w/v) was used as the gelling agent for both media, and the pH was adjusted to 5.8 before autoclaving at 121°C for 20 min.

Cultures were maintained in a growth room with a 16-h-light/8-h-dark photoperiod (45–50 µE m^{-2}s^{-1}) at 24 ± 1°C.

General strategy for regeneration

The first four apical, expanding leaves from 3-week-old axillary shoots were placed in sterile water and swirled to randomise. Leaves were blotted dry on sterile filter paper, cut transversely three or four times across the midrib without fully separating the segments and cultured with the adaxial side in contact with the regeneration medium.

Regeneration strategy and medium were as described by Petri *et al.* (2008*a*). Briefly, shoot regeneration medium (SRM) consisted of QL macronutrients, Driver and Kuniyuki micronutrients, vitamins and organic compounds (Driver & Kuniyuki, 1984) 3% (w/v) sucrose and 0.7% (w/v) TC Agar (Phytotechnology Laboratories, Shawnee Mission, KS, USA: Cat. No. A175). SRM was supplemented with 9.0 µM thidiazuron (TDZ), 4.0 µM α-naphthaleneacetic acid (NAA) and 9.0 µM 2,4 dichlorophenoxy-acetic acid (2,4-D). After 4 days in the dark, explants were placed in SRM without 2,4-D and supplemented with 60-µM silver thiosulphate (STS).

The medium was adjusted with KOH to pH 5.7, autoclaved at 121°C for 20 min and then dispensed into 8.5 cm × 1.5 cm sterile plastic Petri dishes (∼25 mL each).

After explants were positioned on the medium, the dishes were sealed with Parafilm®, and incubated in the dark at 24 ± 1°C. After 2 weeks in the dark, explants were transferred to the light with a 16-h photoperiod (45–50 µE m^{-2} s^{-1}) at 24 ± 1°C.

Leaf explants were maintained in the same Petri dish (not transferred to fresh medium) during the entire experiment. This shoot regeneration protocol was followed, with the modifications indicated below, for all of the experimentation. Adventitious shoot regeneration was evaluated weekly, starting 3 weeks after the leaf explants were placed on the regeneration medium and continuing until adventitious buds stopped appearing for 2 weeks in a row, approximately 9–10 weeks from the beginning of the experiment, at which point, final data were collected on the shoot regeneration frequencies and yields.

Effect of the shoot proliferation medium

Leaf explants from axillary shoots cultured for 3 weeks on fresh JS or SGM shoot multiplication media were collected and placed on SRM for subsequent regeneration.

Effect of the gelling agent

Leaves from shoots maintained in SGM were cultured on SRM with 0.7% (w/v) TC Agar (Phytotechnology Laboratories, Shawnee Mission, KS, USA: Cat. No. A175), 0.7% (w/v) Bacto agar (Voigt Global Distribution Inc., Lawrence, KS, USA: Cat. No. 214010) or 0.45% (w/v) Agargel™ (Sigma-Aldrich, St. Louis, MO, USA: Cat. No. A3301). Agargel™ is a blend of agar and gellan gum (Sigma Phytagel™).

All the three gelling agents were added at the concentration recommended by the manufacturers for plant tissue culture, and this resulted in similar apparent media firmness.

Effect of the dark period

Leaves from shoots cultured in SGM were cultured on SRM under four light or dark treatments: a 16-h photoperiod (45–50 µE m^{-2} s^{-1}) at 24 ± 1°C or complete darkness for 1, 2 or 3 weeks (at 24 ± 1°C) before transfer to a 16-h photoperiod (45–50 µE m^{-2} s^{-1}) at 24 ± 1°C.

Effect of growth regulator concentrations

Leaves from shoots cultured in SGM were placed on SRM with the following combinations and concentrations of growth regulators: 15.0 µM TDZ with 6.0 or 4.0 µM NAA, 12.0 µM TDZ with 6.0 or 4.0 µM NAA, 9.0 µM TDZ with 4.0 or 2.0 µM NAA and 4.5 µM TDZ with 4.0 or 2.0 µM NAA. These combinations were designed to test a range of cytokinin:auxin ratios in the SRM (approximately from 4:1 to 1:1), at different concentration levels.

Bacto agar (0.7%) was used as the gelling agent, and leaves were cultured in the dark for 1 week before transfer to the light.

Effect of ethylene inhibitor (STS)

Leaves from shoots cultured in SGM were cultured on SRM, solidified with 0.7% Bacto agar. After 4 days, when leaf explants were transferred to SRM without 2,4-D, SRM was supplemented with 0, 30, 60 or 120 µM STS.

STS stock solution was prepared by mixing a 0.465 mM AgNO$_3$ solution with a 1.865 mM NaS$_2$O$_3$ solution (ratio 1:4), following procedures described by Burgos & Alburquerque (2003). STS was added to the culture medium prior to autoclaving.

Isolation of regenerated buds, rooting and acclimatisation

When regenerated buds reached approximately 0.5 cm in diameter they were isolated from the leaf explants with a small portion of the callus underneath and placed in SGM. When adventitious shoots reached 2–3 cm long, they were transferred to rooting medium (RM), as described by Gonzalez-Padilla et al. (2003). The basal RM consisted of half of the strength of MS salts, with SGM organics (vitamins and sucrose), 0.1 µM kinetin and 5.0 µM NAA. Different modifications of RM were tested: full-strength MS salts or addition of 100 mg L^{-1} phloroglucinol (PG) or addition of 2.5 µM IBA instead of 5.0 µM NAA. Bacto agar at 0.7% (w/v) was used as the gelling agent. Shoots were cultured under a 16-h-light/8-h-dark photoperiod (45–50 µE m^{-2} s^{-1}) at 24 ± 1°C. Roots appeared after 3–5 weeks, and when roots reached approximately 1-cm long, shoots were ready for acclimatisation.

For acclimatisation, procedures described by Petri et al. (2008b) were followed. Briefly, shoots rooted in RM were washed in sterile water to eliminate agar residues, and then transferred to 7.6-cm^2 peat pots containing Metro-Mix 510 potting medium (SUNGRO Horticulture, Bellevue, WA, USA). The potted plantlets were introduced into zipper-seal plastic bags which were sealed and maintained completely closed in the tissue culture growth room with a 16-h-light/8-h-dark photoperiod (45–50 µE m^{-2} s^{-1}) at 24 ± 1°C. After 2 weeks, the plastic bags were fully opened. Following 2 weeks in open plastic bags, during which time plants were watered with deionised water as needed, the plants were sprayed with an aqueous solution (1:20, v/v) of Anti Stress 2000 (Polymer Ag, Fresno, CA, USA) and cultured under ambient greenhouse conditions of 12–16 h photoperiod and temperatures ranging from 22°C to 26°C.

Experimental design and data analysis

At least five Petri plates were evaluated for each treatment, each plate containing seven leaves. Experiments were repeated twice. For the rooting studies, experiments were repeated three times with a minimum of 10 shoots per treatment.

Regeneration and rooting percentages and number of shoots per regenerating explant were compared with maximum likelihood ANOVA and, when necessary, specific contrasts of maximum likelihood were designed (SAS, 1988). Bonferroni LSD means procedure was used for mean values separation following ANOVA (SAS, 1988).

Results

Although shoot multiplication data were not collected, observations indicated that the rates of culture growth were equivalent across both micropropagation media (JS and SGM) with about two to three shoots being produced per original shoot per 3-week culture period. However, the shoots cultured on JS medium were clearly shorter and had smaller leaf size and were significantly ($P < 0.001$) less competent for shoot regeneration under the experimental conditions employed. Less than 5% of the leaf explants from the shoot cultured in JS medium produced shoots ($1.4 \pm 1.4\%$) compared with mean shoot regeneration frequencies of $51.4 \pm 8.4\%$ for the leaves of shoots cultured in SGM.

After 2 weeks in the dark, leaf explants enlarged and showed some bleaching. Calli appeared on the cut edges and midribs or in association with vascular tissue (Fig. 1a). Buds started appearing after 4 weeks from the beginning of the experiment (Fig. 1b) and after a total of 8–9 weeks no additional buds regenerated. Buds developed from calli and appeared from both adaxial and abaxial sides of the explants (Fig. 1b and Fig. 1c). Occasionally buds formed in clusters (Fig. 1d). Bud viability was high after isolation and placement on SGM (83.7% of the isolated buds survived and elongated).

Regeneration rates were significantly affected by the TDZ concentration ($P < 0.01$), but not by NAA. Separation of means by the Bonferroni LSD method revealed that highest regeneration rates were obtained with 15.0 and 12.0 µM TDZ (Table 1), although often, when 15.0 µM TDZ was added to SRM, buds appeared chlorotic (Fig. 1e). The number of shoots per regenerating explant was not significantly affected by growth regulator concentrations (Table 1).

The type of gelling agent also showed a significant influence on regeneration ($P < 0.01$). Although, regeneration rates were similar with TC agar or Agargel^{TM}, regeneration was increased almost threefold when Bacto agar was used (Fig. 2).

'Improved French' leaves, incubated under a 16-h photoperiod or in the dark for 1 week before transfer to the 16-h photoperiod, showed significantly more organogenic activity ($P < 0.01$) than when explants were cultured in the dark for longer than 1 week (Fig. 3). Although calli appeared for all treatments, an increase in callus formation was observed as the dark period length was increased.

Specific statistical contrasts that compared each STS treatment with the non-STS control showed significant differences in the regeneration percentages when 60 µM ($P < 0.05$) or 120 µM ($P < 0.01$) was added to SRM (Fig. 4). Buds often appeared chlorotic when the medium
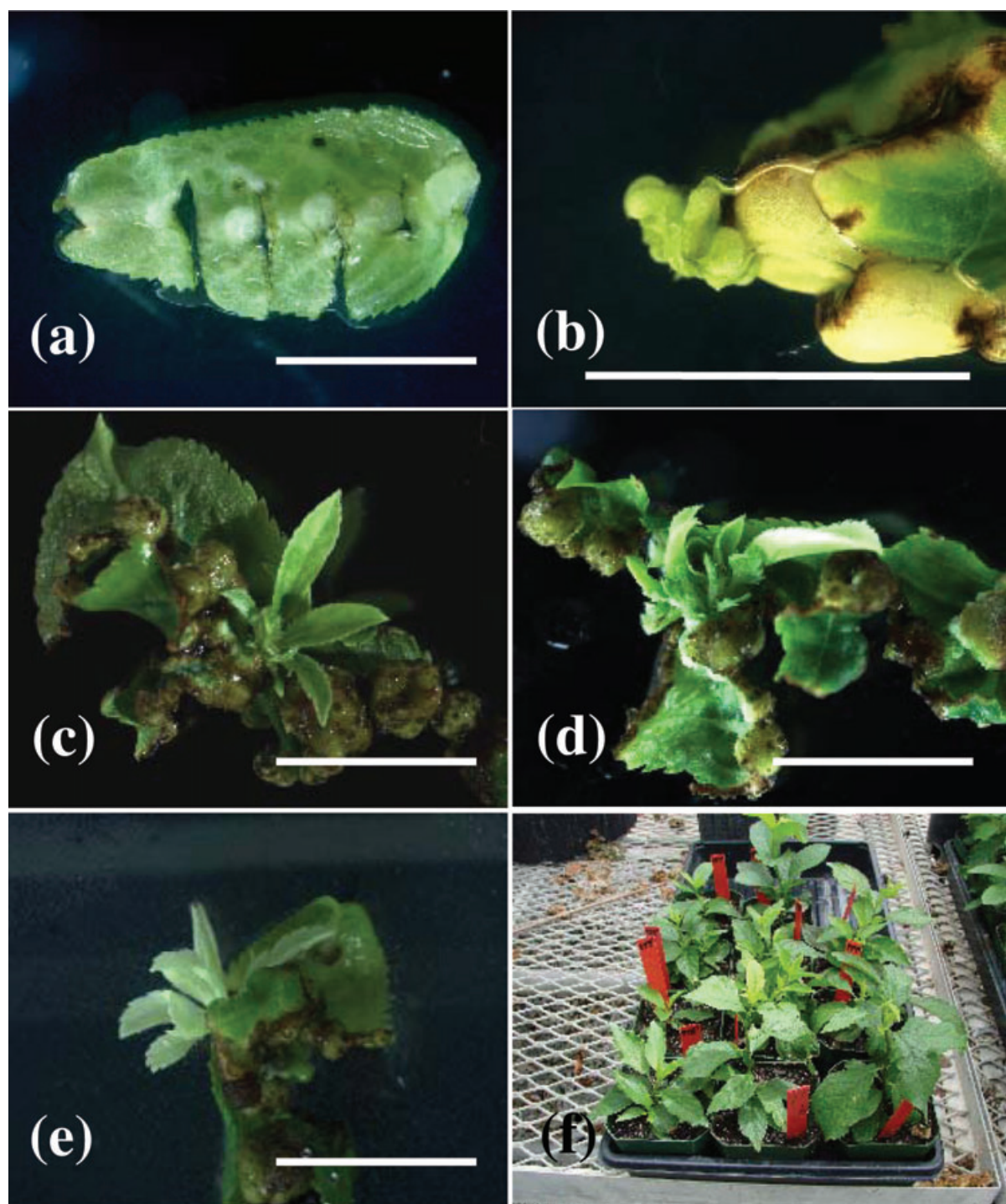
Figure 1 (a) Leaf explant when transferred to light after 2 weeks of culture in dark. (b) Bud regenerating from callus tissue at 4 weeks from the beginning of the experiment. (c) Regenerating shoot from 'Improved French' leaf. (d) Cluster of shoots. (e) Regenerating shoot from leaves cultivated in medium with 15.0 μM thidiazuron. (f) 'Improved French' regenerated plantlets cultivated in a greenhouse. Bars indicate 1 cm. Nine to ten weeks were allotted for shoot regeneration. The explants were maintained on the same plates of culture medium for the entire duration of the experiment excluding the transfer to 2,4 dichlorophenoxy-acetic acid free media at the 4-day point (see Materials and methods).

Table 1 Effect of thidiazuron (TDZ) and naphtaleneacetic acid (NAA) on adventitious shoot regeneration from 'Improved French' leaf explants[a]

Growth Regulators (µM)			Percentage of Regenerating Leaves	Number of Shoots per Regenerating Leaf
TDZ	NAA	Number of Explants	(mean ± SE)	(mean ± SE)
15.0	6.0	70	48.6 ± 6.0 ab	1.58 ± 0.17
15.0	4.0	70	64.3 ± 5.7 a	1.62 ± 0.11
12.0	6.0	70	47.1 ± 6.0 ab	1.78 ± 0.17
12.0	4.0	70	55.7 ± 5.9 a	1.72 ± 0.14
9.0	4.0	140	36.4 ± 4.1 bc	1.57 ± 0.13
9.0	2.0	70	45.7 ± 6.0 ab	1.87 ± 0.19
4.5	4.0	70	20.0 ± 4.8 c	1.57 ± 0.27
4.5	2.0	70	30.0 ± 5.5 bc	1.29 ± 0.12

[a]Any two mean values with no letter in common are significantly ($P = 0.05$) different by the Bonferroni LSD method. 2,4 Dichlorophenoxy-acetic acid (9.0 µM) was added to the medium during the first 4 days in culture, and then explants were transferred to medium without 2,4 dichlorophenoxy-acetic acid and supplemented with 60 µM silver thiosulphate. Nine to ten weeks was allotted for shoot regeneration. The explants were maintained on the same plates of culture medium for the entire duration of the experiment excluding the transfer to 2,4 dichlorophenoxy-acetic acid free media at the 4-day point (see Materials and methods)
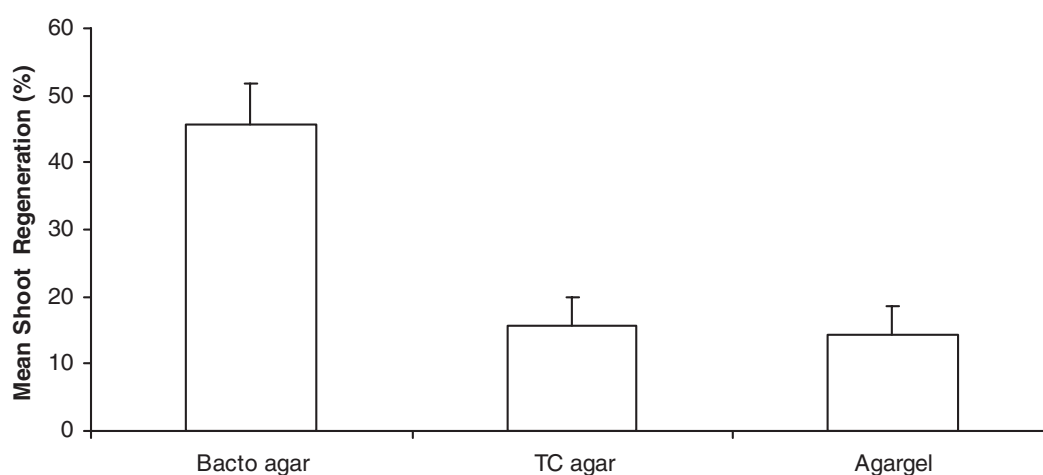

Figure 2 Effect of different gelling agents on adventitious shoot regeneration from leaf explants of plum convertible 'Improved French'. A total of 210 explants were used in this study. Bars indicate standard errors. Nine to ten weeks were allotted for shoot regeneration. The explants were maintained on the same plates of culture medium for the entire duration of the experiment excluding the transfer to 2,4 dichlorophenoxy-acetic acid free media at the 4-day point (see Materials and methods).

was supplemented with 120 µM STS, as well as when 15.0 µM TDZ was applied (Fig. 1e).

Rooting efficiency was very low when RM, as described by Gonzalez-Padilla *et al.* (2003), was used (Table 2). The addition of full-strength MS salts or 100 mg L^{-1} PG to RM increased rooting efficiency significantly for 'Improved French' shoots (Table 2). Substitution of 5.0 µM NAA by 2.5 µM IBA did not improve rooting rates compared to RM. No visual differences between the root system (length, diameter, etc.) of shoots rooted in the different RM modifications were observed. All 17 shoots that were used to test the acclimatisation step survived (Fig. 1f).

Discussion

Our results agree with the previous reports showing that the medium in which shoots (used as the source of explants) were cultured had a significant influence on subsequent regeneration (Antonelli & Druart, 1990; Burgos & Alburquerque, 2003). In this study, significant differences in regeneration capacity of leaves were found depending on the shoot proliferation medium used. The main difference between the two shoot proliferation media used was the concentration of BA, almost threefold lower than that used in the JS medium (3.0 µM in SGM vs 8.89 µM). This may explain why axillary shoots were, on average, shorter with smaller leaves when shoots were
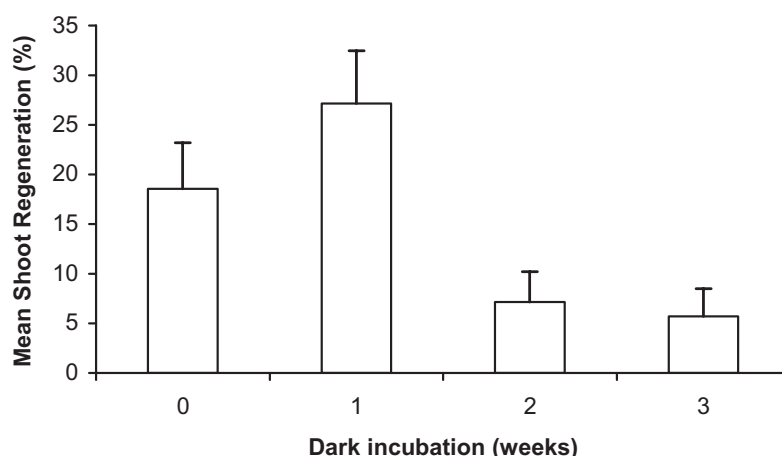
Figure 3 Influence of dark period on the regeneration percentages from leaves of 'Improved French'. A total of 280 explants were used in this study. Bars indicate standard errors. Nine to ten weeks were allotted for shoot regeneration. The explants were maintained on the same plates of culture medium for the entire duration of the experiment excluding the transfer to 2,4 dichlorophenoxy-acetic acid free media at the 4-day point (see Materials and methods).
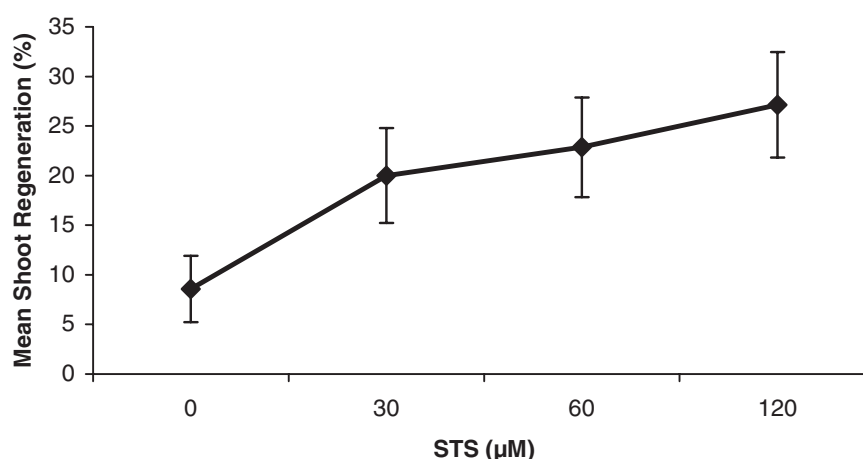

Figure 4 Effect of different concentrations of silver thiosulphate on adventitious regeneration from 'Improved French' leaf explants. Regeneration was calculated as the average of 70 explants for each treatment. Vertical bars represent standard errors. Nine to ten weeks were allotted for shoot regeneration. The explants were maintained on the same plates of culture medium for the entire duration of the experiment excluding the transfer to 2,4 dichlorophenoxy-acetic acid free media at the 4-day point (see Materials and methods).

cultured in JS medium compared with shoots cultured in SGM. This agrees with results obtained in *Prunus canescens* where shoot regeneration occurred satisfactorily on leaves collected from shoots grown in an elongation medium, without BA, but not on those taken from shoots cultured in a proliferation medium with 4.4-μM BA (Antonelli & Druart, 1990). In addition, significant increases in shoot regeneration from apricot leaves were obtained when shoots were cultured in a proliferation medium with a lower BA concentration (1.78 vs 3.31 μM) (Burgos & Alburquerque, 2003). The authors hypothesised that

propagation media affect the physiological status of the plant material, an important factor in regeneration.

This TDZ-based regeneration medium produced good results from 'Improved French' leaf explants. TDZ has been used successfully for plum regeneration from leaf explants, ranging from 12.5% to 65.5% for the cultivars 'Damas de Toulouse' (Escalettes & Dosba, 1993) or 'Wegierka Zwykla' (Nowak *et al*., 2004) respectively. Although high regeneration efficiency has been reported when BA was used in the regeneration medium (80%) for the plum cultivar 'Startovaya' (Mikhailov & Dolgov, 2007), for the cultivar 'Damas de Toulouse'

Table 2 Rooting of 'Improved French' *in vitro* shoots using different media composition[a]

Rooting Medium (RM)	Number of Shoots	Rooting Rate (%)	Number of Roots per Shoot
RM[b]	48	20.8 b	2.0
RM full MS Salts	30	40.0 a	2.3
RM 100 mg L^{-1} PG	30	53.3 a	2.2
RM 2.5 μM IBA	30	20.0 b	1.3

IBA, indole-3-butyric acid; MS, Murashige and Skoog salt; PG, phloroglucinol.

[a] Any two mean values with no letter in common are significantly ($P = 0.05$) different by the Bonferroni LSD method.

[b] As described by Gonzalez-Padilla *et al*. (2003). See Materials and methods for composition.

no adventitious regeneration was achieved when BA was added, and only TDZ promoted shoot regeneration (Escalettes & Dosba, 1993). TDZ has been more effective than BA for inducing shoot regeneration from leaves of other *Prunus* species such as *P. armeniaca* (Pérez-Tornero *et al*., 2000), *P. avium* (Bhagwat & Lane, 2004) and *P. serotina* (Hammatt & Grant, 1998; Espinosa *et al*., 2006). Pérez-Tornero *et al*. (2000) also found that regeneration percentages were affected only by the TDZ concentration, but not by NAA.

The optimum TDZ concentration for plum has been shown to vary depending on the cultivar from 3.0 μM (Escalettes & Dosba, 1993) to 7.5 μM (Nowak *et al*., 2004). Even though regeneration rates were slightly higher when 15.0 μM TDZ was added to SRM, 12.0 μM TDZ (with 4.0 μM NAA) was the optimum concentration as regenerated buds appeared healthier. While cytokinins are well known for antisenescence activity, it has been demonstrated that 13.0 μM BA induced programmed cell death in carrot and *Arabidopsis* cell-suspension cultures and in *Arabidopsis* plants, probably by accelerating the senescence process (Carimi *et al*., 2003). Low bud viability (Petri *et al*., 2008*a*) or production of swollen or fasciated shoots (Huetteman & Preece, 1993; Bosela & Michler, 2008) has been reported in other woody plants, related to high TDZ levels.

Gelling agents have been shown to influence growth *in vitro*, depending on the type (Chevreau *et al*., 1997), manufacturer and concentration (Debergh, 1983). Also, levels of impurities differ, and the nutrient status of the medium may be affected (Mackay & Kitto, 1988). Previous studies in apple (Welander & Maheswaran, 1992), pear (Chevreau *et al*., 1997) and apricot (Pérez-Tornero *et al*., 2000; Burgos & Alburquerque, 2003) demonstrated that gelling agents significantly affect regeneration. The three gelling agents used in this study have different ionic composition. In general, agars contain

less mineral impurity than Gellan gum, a component of Agargel^{TM}. In addition, TC Agar, a purified grade agar, would be expected to contain less mineral nutrient impurity than Bacto agar. This may explain the present results. The use of Gellan gum increased the regeneration rate for all genotypes tested in apple (41% vs 14% with agar) (Welander & Maheswaran, 1992), and in pear, reaching 95% for one cultivar versus 52% with agar (Chevreau *et al*., 1997). In another report, Gellan gum significantly decreased adventitious regeneration in apricot, compared with agar and Agargel^{TM} (Pérez-Tornero *et al*., 2000). The purest gelling agent assayed, purified agar, increased regeneration from 20% to 40%, but only for one of the two apricot cultivars tested (Burgos & Alburquerque, 2003). According to the manufacturer, Bacto agar has been optimised for calcium and magnesium contents, and detrimental ions such as iron and copper have been reduced for bacterial culture (http://www.bd.com/ds/technicalCenter/inserts/Agars.pdf). As the effect of gelling agent seems to be genotype dependent, Bacto agar was more appropriate for 'Improved French' than the other gelling agents tested.

A dark period has been shown to be a key factor in organogenesis for different fruit tree species, with the optimum dark period varying between 1 and 4 weeks (Leblay *et al*., 1990; Korban *et al*., 1992; Miguel *et al*., 1996; Pérez-Tornero *et al*., 2000; Gentile *et al*., 2002; Espinosa *et al*., 2006). In apricot, higher regeneration occurred with 2 or 3 weeks (30%) compared with 1 or 4 weeks (<10%) of dark incubation before light treatment (Pérez-Tornero *et al*., 2000). Our results do not support the need for a longer dark period and follow previous results for *P. domestica* hypocotyl explants (Mante *et al*., 1989; Gonzalez-Padilla *et al*., 2003). With leaves explants, no dark or a 1-week dark period produced the best results. It has been reported that the dark period can influence endogenous levels of growth regulators such as indole-3-acetic acid (IAA) (Lopez-Carbonell *et al*., 1992). Some reports attributed the positive effect observed on adventitious shoot regeneration of the dark induction to an increase in the IAA levels during this period which then interacted with exogenously applied growth regulators (Korban *et al*., 1992; Miguel *et al*., 1996). The use of high cytokinin:auxin ratios induces shoot organogenesis (Hamill, 1993). The increase in the endogenous auxin levels during the dark period could have a detrimental effect on shoot regeneration from 'Improved French' leaf explants cultured in SRM, because it could lead to a low cytokinin:auxin ratio.

Beneficial effects of ethylene inhibitors on organogenesis have been widely reported (Songstad *et al*., 1988; Chi *et al*., 1990; Chraibi *et al*., 1991; Ma *et al*., 1998; Bais *et al*., 2001), but only a few previous reports (Escalettes &

Dosba, 1993; Burgos & Alburquerque, 2003; Petri *et al.*, 2005; Liu & Pijut, 2008) describe the effect of ethylene inhibitors, including Ag^+, on organogenesis from *Prunus*. In all these reports, Ag^+ significantly stimulated adventitious regeneration.

The ethylene inhibiting effect of STS (Ag^+) in plant tissues is believed to act through the attachment of Ag^+ to ethylene binding sites (Beyer, 1979). The optimum STS level has been shown to be genotype dependent, varying between 30 and 60 µM, for two apricot cultivars (Burgos & Alburquerque, 2003), or between 60 and 80 µM, for two black cherry genotypes (Liu & Pijut, 2008). We found 120 µM as the optimum concentration for 'Improved French'. In future studies, higher STS concentrations should be tested in order to determinate if the optimum concentration was 120 µM or higher, but the chlorotic aspect observed in buds that regenerated in medium with 120 µM STS may indicate proximity to toxic levels and higher concentrations might have a detrimental effect on adventitious shoot regeneration.

Our results suggest that the rooting process is genotype dependent because 91% of shoots of the plum cultivar 'Bluebyrd' rooted when placed in RM (Gonzalez-Padilla *et al.*, 2003) versus only 21% of the 'Improved French' shoots evaluated in this study. PG is a rooting cofactor that has been previously reported to stimulate rhizogenesis in other fruit tree species (Jones & Hatfield, 1976; James & Thurbon, 1981; Hammerschlag *et al.*, 1987). In peach, the addition of 100 mg L^{-1} PG significantly increased rooting efficiency for all genotypes tested, that is 67% and 100% with PG versus 6% and 35% without PG for the cultivars 'Redhaven' and 'Jerseyqueen' respectively (Hammerschlag *et al.*, 1987). This agrees with our results showing that PG significantly increased root formation.

Conclusions

Our results, together with previous reports, indicate that adventitious shoot regeneration from leaf explants was highly genotype dependent. Several factors and conditions must be studied in order to establish an efficient protocol for each cultivar. The best results for 'Improved French' were obtained when 3-week old leaves, from shoots cultured in SGM proliferation medium, were collected and placed on SRM supplemented with 9.0 µM 2,4-D (only during the first 4 days), 12.0 µM TDZ, 4.0 µM NAA, 120 µM STS, 0.7% Bacto agar, and cultured in the dark for 1 week before transfer to a 16-h photoperiod. This study provides the possibility for GE of the most important dried plum cultivar in the USA.

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